AN ABSTRACT OF THE THESIS OF

Jessica L. Flesher for the degree of Honors Baccalaureate of Science in Biology presented on May 13, 2013. Title: Is there a change in expression of apoptosis and autophagy genes in *Aiptasia* sp. after thermal stress?

Abstract approved:

Virginia Weis

Corals form the foundation for coral reef ecosystems and contain symbiotic dinoflagellates which greatly contribute to reef primary productivity. Loss of dinoflagellates from animal (host) cells results in cnidarian bleaching which leads to decreased coral fitness, and reef deterioration. Elevated temperature, caused by global warming, is the primary environmental stressor that causes bleaching, but the cellular mechanisms leading to the collapse of the symbiosis are not fully understood. Two cellular pathways hypothesized to result in bleaching are host cell apoptosis and autophagy. To determine if there are changes in expression of apoptosis- and autophagy-specific genes during thermal stress, quantitative PCR was performed on cDNA of the symbiotic anemone *Aiptasia* sp. subjected to elevated temperatures. Acasp and LC3, part of the apoptosis and autophagy cascades, respectively, were used as markers. There was no change in acasp expression with elevated temperature, a result consistent with vertebrate systems. LC3 expression had a downward trend with increasing temperature, indicating that autophagy may not be the dominant bleaching mechanism. Additionally, the function of *Aiptasia* LC3 was compared with human LC3. Mouse cells transfected with *Aiptasia* LC3 displayed similar autophagosome localization patterns to those containing human LC3, suggesting the presence of autophagic machinery in *Aiptasia*.

Key Words: *Aiptasia*, apoptosis, autophagy, bleaching, *Symbiodinium*
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Is there a change in expression of apoptosis and autophagy genes in *Aiptasia* sp. after thermal stress?

by

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Jessica L. Flesher, Author

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TABLE OF CONTENTS

LIST OF FIGURES

Page

DEDICATION

I would like to dedicate this thesis to my sister, Susanna Flesher, who has always been an inspiration for me to move forward; my brothers, Andrew and Daniel Flesher, who keep me fighting; and my parents, David and Sarah Flesher, who pushed me to succeed every step of the way.

Is there a change in expression of apoptosis and autophagy genes in *Aiptasia* sp. after thermal stress?

Introduction

Reef-building corals are the foundation of coral reef ecosystems. Corals provide a biogenic habitat for thousands of species of algae, fish, and invertebrates as well as dinoflagellate algal symbionts, *Symbiodinium* spp., that reside in the gastrodermal tissue of individual coral polyps (Davy et al. 2012). The host coral provides the dinoflagellates with shelter and inorganic nitrogen and carbon for photosynthesis (Davy et al. 2012), allowing the dinoflagellates to remove carbon dioxide and metabolic waste products from the host cell (Yellowlees et al. 2008). Excess photosynthetic products from the symbionts are translocated to the host cell (Davy et al. 2012), and the fixed carbon contributes to the high primary productivity of coral reefs (Davy et al. 2012, Lesser 2011). In addition to these high levels of productivity, this mutualism between healthy corals and dinoflagellates is responsible for increasing the rate of coral calcification (Davy et al. 2012, Weis and Allemand 2009) which creates the three-dimensional structure for the coral ecosystem.

When the healthy symbiosis breaks down, the result is cnidarian bleaching, the loss of the dinoflagellates or pigments from host cells. Extensive loss of the golden-brown dinoflagellates from host tissues causes the coral to lose color, appearing pale. In laboratory settings, bleaching can be induced by temperature extremes, high irradiance, prolonged darkness, and pathogens (Douglas 2003). However, large-scale bleaching of

coral reefs has been attributed to elevated temperature often in combination with high solar radiation (Douglas 2003, Venn et al. 2008), environmental stressors resulting from climate change (Weis and Allemand 2009). Duration and intensity of stress affect the levels of bleaching and mortality (Lesser 2011). Both a large increase in temperature or a moderate increase of 1-2°C, especially when combined with other stressors, typically cause bleaching (Weis 2008). Bleached corals can recover if the triggers are removed and environmental conditions return to normal (Lesser 2011), but bleaching reduces growth rates, reproduction, and increases susceptibility to disease and mechanical damage (Venn et al. 2008).

Though the cellular mechanisms for bleaching are not yet fully understood, two hypothesized mechanisms for bleaching are host cell apoptosis and *in situ* degradation in the form of autophagy (Weis 2008). Apoptosis, or programmed cell death is a highly-conserved set of cellular pathways found in animals, plants, and fungi (Dunn et al. 2006) used to eliminate dysfunctional or diseased cells (Dunn et al. 2007a). Both intrinsic and extrinsic pro-death signals can lead to the activation of cysteine-dependent aspartate-specific proteases, or caspases, present in cells as their inactive zymogens (Kitazumi and Tsukahara 2011). Caspases form a cascade, where the activated proteases cleave additional caspases and other specific proteins at aspartic acid residues (Hyman and Yaun 2012, Ola et al. 2011). The culmination of the cellular cascade results in cell death via apoptosis (Hyman and Yaun 2012).

In symbiotic cnidarians, host cell apoptosis is the regulated degradation of the host cell and the symbiont is released either whole or partially degraded (Weis 2008). For example, host cell death by apoptosis was evident in localized regions of the

gastrodermis of the coral *Acropora aspera* incubated at 30°C, and present in all tissues when incubated at 32°C (Ainsworth et al. 2008). In sea anemones placed under thermal stress, initial apoptotic cell death gave way to slower uncontrolled, necrotic cell death pathways (Dunn et al. 2004). Caspase homologues have been identified in cnidarians including the hydroid *Hydra vulgarus*, and the anthozonan sea anemones *Aiptasia* sp. (Dunn et al. 2006) and *Anemonia viridis* (Richier et al. 2006). The *Aiptasia* caspase sequence, acasp, shares required structural similarities with functional mammalian caspases (Dunn et al. 2006).

Autophagy is another conserved regulatory mechanism found in a range of species from yeast to humans (Nakatogawa et al. 2009, Shpilka 2011). To maintain cell homeostasis, autophagy is used to remove unwanted protein aggregates as well as excess or damaged organelles under nutrient starvation, during differentiation and development, and in response to pathogenic infections (Weis 2008). During autophagy, unwanted cellular components and cytosol are isolated within an autophagosome, a double membrane-bound vesicle, which forms around the cellular components (Nakatogawa et al. 2009). The autophagosome then fuses with a lysosome and the contents are degraded (Nakatogawa et al. 2009). In mammals, the microtubule-associated protein 1 light chain 3 (LC3) localizes to autophagosome membranes (Kabeya et al. 2000) by lipid conjugation to a phosphatidylethanolamine (Kim and Lemasters 2011), and is required for autophagosome formation and elongation (Nakatogawa et al. 2009, Shpilka 2011). After the autophagosome has fused with the lysosome, membrane-bound LC3 is either released or degraded (Kim and Lemasters 2011). Since LC3 is bound to autophagosomes

from formation to fusion with lysosomes, the presence of LC3 is a diagnostic for autophagy (Young et al. 2011).

Autophagy is another mechanism hypothesized to play a role in cnidarian bleaching whereby an autophagosome forms around the targeted endosymbiotic dinoflagellate, fuses with a lysosome, and degrades the symbiont. Anemones treated with an autophagy inducer showed increased levels of bleaching compared to control animals (Dunn et al. 2007a). Widespread autophagy was also documented during transitory fusion in the hydra *Hydractinia symbiologicarpus* (Buss et al. 2012). Additionally, LC3 homologues have been identified in the anemone *Nematostella vectensis* (Sphilka et al. 2011) and in *Hydra* (Chera et al. 2009). In a study of both apoptosis and autophagy during temperature stress in *Aiptasia*, Dunn et. al. (2007a), found that when one cellular pathway, either apoptosis or autophagy, was inhibited, there was no significant change in the bleaching response at an elevated temperature, but when both pathways were inhibited simultaneously levels of bleaching were greatly reduced. This suggests that multiple interconnected pathways may be responsible for cnidarian bleaching.

To determine if apoptotic and/or autophagic pathways driving cnidarian bleaching are controlled at the transcriptional level, quantitative reverse-transcriptase PCR was used to quantify the gene expression of an apoptosis- and an autophagy-specific sequence after thermal stress in the symbiotic anemone *Aiptasia*. The symbiosis between *Aiptasia* and *Symbiodinium* is functionally similar to the symbiosis found in corals (Dunn et al. 2004), and is an ideal model system for corals, which are difficult to maintain and manipulate in the laboratory (Lehnert et al. 2012). The previously identified acasp was used as a

marker for apoptosis (Dunn et al. 2006), and an *Aiptasia* LC3 homologue was identified, isolated and used as a marker for autophagy. The expression of these sequences was quantified for anemones incubated at control and elevated temperatures for varying lengths of time to trigger a bleaching response. Additionally, to determine if *Aiptasia* LC3 has similar functionality to human LC3, both sequences were tagged and transfected into mouse cells to look for evidence of autophagosome formation.

Materials and Methods

Anemone culture and treatment

Sixty *Aiptasia* were placed in filtered artificial seawater (FASW) and separated into four treatment groups of fifteen. A schematic of the following experimental design is shown in Figure 1. Each treatment group was placed in a separate 6 well-plate with three anemones per well. Animals were incubated at 25 ± 0.5 °C on a 12 hour light/dark cycle for a two day acclimation period. Following a water change, anemones were placed at one of four treatment temperatures: 25°C control, 27°C, 30°C, and 33°C. The control anemones remained in the original incubator and anemones destined for elevated temperature stress were moved to a separate incubator on a 12 hour light/dark cycle with 1°C fluctuation around the treatment temperature. The FASW was changed every other day with preheated water. At each of five time points (12, 24, 48, 96, 168 hours), three anemones were removed from each plate, snap frozen in liquid nitrogen, and stored at -80°C.

RNA extraction, purification, DNase treatment, and cDNA conversion

RNA extractions were performed using a Trizol/RNeasy kit (Qiagen) hybrid protocol. After the Trizol steps, RNase Zap (Sigma) was used to prevent contamination by RNases. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. Some samples displayed low 260/230 ratio indicating the presence of contaminating phenol or guanidine which are present in Trizol, therefore RNA purification was performed. To the total RNA volume, 10% 3M sodium acetate, 5%

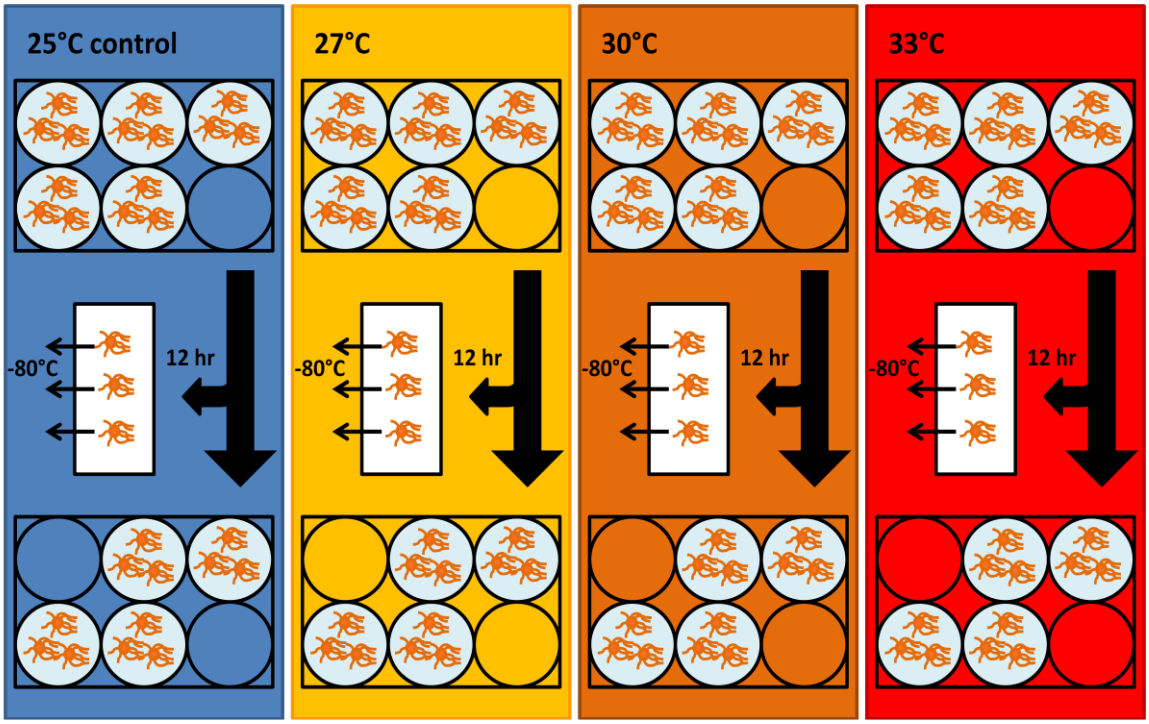

Figure 1. Incubation of *Aiptasia* at control and elevated temperatures. Sixty anemones were divided into four temperature treatment groups of fifteen anemones. Three anemones were removed from each treatment group at the 12 hour time point. Three additional anemones at each temperature were removed at the 24, 48, 96, and 168 hour time points.

glycogen and 2.5 volumes of 100% ethanol was added and incubated at -80°C overnight. RNA was centrifuged at 9500RPM for 20 minutes at 4°C, 250µL of 70% ethanol was used to wash the pellet. After an additional centrifugation of 5 minutes, the RNA was resuspended in 20µL of nuclease free water. RNA samples were treated with 1 unit of DNase treated using DNA-*free* (Ambion). Total RNA (750ng) was reverse transcribed in a 20µL reaction using 1µL of 50µM oligo$(dT)_{20}$ and 200 U SuperScript III reverse transcriptase (Qiagen), according to manufacturer's instructions. The resulting cDNA was then treated with 1µL of RNase H to remove the RNA template and diluted to 50µL of cDNA.

Identification of target sequences and cloning

The human microtubule-associated protein 1 light chain 3 (LC3) sequence was blasted against an *Aiptasia* transcriptome (Lehnert et al. 2012) to identify an *Aiptasia* LC3 homologue. Initial primers were designed using Geneious (Biomatters) to incorporate as much of the *Aiptasia* LC3 sequence as possible and ordered through Integrated DNA Technologies. Primers were also designed for acasp, a previously identified caspase ortholog in *Aiptasia* (Dunn et al. 2006). Sequences of interest were amplified using PCR and inserted into the pGEM – T Easy Vector (Promega). DH5α *Escherichia coli* cells were transformed with the vector, grown on ampicillin LB agar plates, and incubated at 37°C overnight. Colonies containing the sequences of interest in the vector were isolated and grown in ampicillin LB at 37°C, shaking at 200rpm overnight. Plasmids were purified using QIAprep Spin Kit (Qiagen) and sequenced at the Center for Genome Research & Biocomputing at Oregon State University. Quantitative reverse transcriptase PCR (qRT-PCR) primers for both sequences were developed for a 100bp amplicon, with 40-60% GC content, and Tm close to 60°C. Primers were checked for accuracy following the same cloning procedure above.

Quantitative reverse transcriptase – PCR

Samples for qRT-PCR were prepared by mixing 0.5µL of template cDNA, with 1µL of each 5µM primer, 10µL of SYBR Green (Applied Biosystems), with nuclease free water to total 20µL in volume. For relative quantification of the acasp and the LC3-like sequence, three reference genes were used: L10, PABP, and GAPDH (Kitchen and Poole in preparation). Primers for the sequences of interest and reference genes are

Table 1. Forward and reverse primers for *Aiptasia* cDNA used in qRT-PCR analysis and isolation of full LC3 sequence for transfection. Reference genes are indicated with (*).

Gene	Forward Primer	Reverse Primer
qRT-PCR		
acasp	5'-GCACCTCCAGCTGACAAGCGT-3'	5'-TCAGCAATGGACTGGATAAAC-3'
LC3	GCAGAAGAGACGAAGTAGCCG	TTGTGAGCTCCTGGGGCACT
L10*	ACGTTTCTGCCGTGGTGTCCC	CGGGCAGCTTCAAGGGCTTCA
PABP*	GTGCAAGGAGGCGGACAGGG	TGGGCTGATTGCGGGTTGCC
GAPDH*	AAGGCTGCTAAGGCCAATCGGC	TCGCACGGTTAAGTCCAAGAC
Transfection		
LC3	AAACTCGAGATGGGAGACAAC	AAACTGCAGTTATCAGCTATT

shown in Table 1. Each combination of cDNA and primers was amplified in triplicate. Using an Applied Biosystems 7500 Fast Real-Time PCR system, the thermocycling conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min; and the dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, and 60°C for 1 min.

Raw data were exported into LinRegPCR (version 12.17) where the baseline fluorescence was determined and subtracted from each sample. Primer efficiency of each individual primer and the Ct values for each sample were determined. GenEx software (MultiD Analyses AB) was used to correct for the primer efficiency. Analyses of acasp and LC3 were performed separately. Using GenEx, individual plates were standardized using a plate reference, technical repeats were averaged, the sequences of interest were normalized to the reference genes, relative quantities were obtained from the maximum value, and a log base 2 was taken of these relative quantities.

Statistical analyses of the relative expression of acasp and LC3 were performed using a two-way ANOVA with R statistical software (version 2.15.2). A *post hoc* pairwise t-test with the Bonferroni correction was also performed on acasp expression at 96 hour time points.

Preparation of EGFP vector containing *Aiptasia* LC3

The EGFP-LC3 vector (Addgene), which contains a human LC3 sequence, was used as a positive control. To engineer an *Aiptasia* LC3 vector, the human sequence was cut out to obtain an empty EGFP vector. Primers were designed to amplify the entire *Aiptasia* sequence from *Aiptasia* cDNA (Table 1). In addition, the primers contained forward and reverse restriction sites for Xho I and Pst I, respectively. First, the sequence was amplified and cloned into the pGEM – T Easy Vector using methods described above. Using Xho I (GIBCO) and Pst I (Promega), *Aiptasia* LC3 sequence was removed from 1000ng of the pGEM – T Easy Vector. One thousand ng of EGFP vector was linearized. Four ratios of linearized EGFP vector to LC3 insert were used for the overnight ligation at 4°C (3:1, 1:1, 1:3, 1:5). The remaining cloning procedure was performed as described above; with the exception that kanamycin was used as the antibiotic instead of ampicillin. Sequences were checked for accuracy and to ensure that the *Aiptasia* LC3 sequence was inserted in the correct reading frame. The vector with *Aiptasia* LC3 used for the transfection was obtained using the 1:5 ratio of vector to insert.

Three versions of the EGFP vector were transformed into bacteria: empty EGFP vector, EGFP containing human LC3, and EGFP containing *Aiptasia* LC3. For each version of the vector, a glycerol stock was made containing 500µL of bacterial culture

and 500µL of glycerol. To isolate a large quantity of each vector, bacteria were incubated overnight at 37°C, shaking at 200rpm in 200mL of LB with kanamycin. Each vector was purified using Plasmid Plus Maxi Kit (QIAGEN), and quantified using the nanodrop, and then stored at -20°C.

Transfection and imaging of mouse cells with human and *Aiptasia* LC3

NIH3T3 mouse cells in a dimethyl sulfoxide (DMSO) solution were pelleted by centrifugation at 1000g for 4 min. Cells were resuspended to be 80% confluent in 1x Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum. Three mL of mouse cells were plated into 35mm glass bottom dishes (InVitro Scientific) and incubated at 37°C and 5% CO_2 overnight. Four µg of vector DNA was inserted into mouse cells using 10µL of Lipofectamine 2000 (Invitrogen) and 500µL of RPMI 1640 Media. Transfection of each vector was repeated in triplicate. The cells were incubated at 37°C and 5% CO_2 for 48 hours. Three images from each plate of cells were acquired with a Zeiss LSM 510 Meta microscope with a 40/0.8 water objective lens (Zeiss, Thornwood, NY, USA). Fluorescence excitation/emission for GFP was 488/505-530nm.

Results

Expression levels of apoptosis and autophagy sequences in *Aiptasia* during thermal stress

The expression of acasp in *Aiptasia* over time at the control and elevated temperature incubations is shown in Figure 2. Expression levels for acasp had a broad range from 0.59 ± 0.13 to 3.29 ± 1.13. There was a high level of variability between the expression levels at all temperatures, and no clear patterns were present. Initially, at the 25°C control temperature there was a large increase in acasp expression from 0.59 ± 0.13 at 12 hours to 3.24 ± 0.14 at 24 hours. Likewise, at 33°C, there was an increase in expression from 0.63 ± 0.09 at 12 hours to 3.29 ± 1.13 at 24 hours. These large increases encompass the range of acasp expression at 25 and 33°C. After these initial increases, at 48 hours the 33°C treatment anemones had a larger decrease in acasp expression than at the control temperature. Anemones treated at 27°C had the most noticeable change in acasp expression between the 48 and 96 hour time points, where the expression decreased from 2.08 ± 0.28 to 1.00 ± 0.41. For animals treated at 30°C, the acasp expression had the largest change in expression from 0.62 ± 0.57 at 96 hours to 2.05 ± 0.53 at 168 hours. These changes in acasp expression, which encompass the range at the middle treatment temperatures, show that the variation at 27 and 30°C was not as large then at the extreme temperatures. At the 96 hour time point, however, the acasp expression levels at 25 and 33°C grouped together; separate from expression levels at 27 and 30°C.

Using a two-way ANOVA both time ($P = 3.65 \times 10^{-7}$) and temperature ($P = 2.25 \times 10^{-4}$) were significant predictors of the expression of acasp. There was also a significant interaction between incubation time and temperature ($P = 1.48 \times 10^{-6}$). Since

grouping appears at the 96 hour time point, a pairwise t-test was run with a Bonferroni correction comparing expression levels for each temperature treatment at 96 hours. The expression of acasp at the control temperature was significantly different from both expression levels at 27 ($P = 0.02$) and 30°C ($P = 0.005$), but not significantly different from acasp expression at 33°C ($P = 0.64$). Likewise, acasp expression at 33°C was significantly different from the expression levels at both 27 ($P = 0.002$) and 30°C ($P = 0.0007$). Between the expression levels at 27 and 30°C there was not a significant difference ($P = 1.00$).

The expression of LC3 in *Aiptasia* over time at the control and elevated incubation temperatures is shown in Figure 3. LC3 expression displayed a downward trend over time at 25 and 33°C. The LC3 expression levels of animals incubated at the control temperature decreased from 2.71 ± 0.97 at 12 hours to 1.67 ± 0.39 at 48 hours. Likewise, values in animals incubated at 33°C decreased from 2.74 ± 0.27 at 12 hours to 1.31 ± 1.14 at 48 hours. At 27°C, there was an increase in LC3 expression from 1.94 ± 1.93 at 24 hours to 2.18 ± 0.61 at 48 hours. An increase in expression of LC3 was also observed at 30°C from 2.62 ± 0.51 at 24 hours to 2.69 ± 0.38 at 48 hours. Using a two-way ANOVA neither time ($P = 0.11$) nor temperature ($P = 0.60$) were significant predictors of the expression of LC3. There also was not a significant interaction between incubation time and temperature ($P = 0.40$).

During the elevated temperature incubation at 33°C after the 96 hour time point the anemones were visibly pale, suggesting that symbionts had been lost from host tissues during the incubation. Symbiont numbers, however, were not quantified.

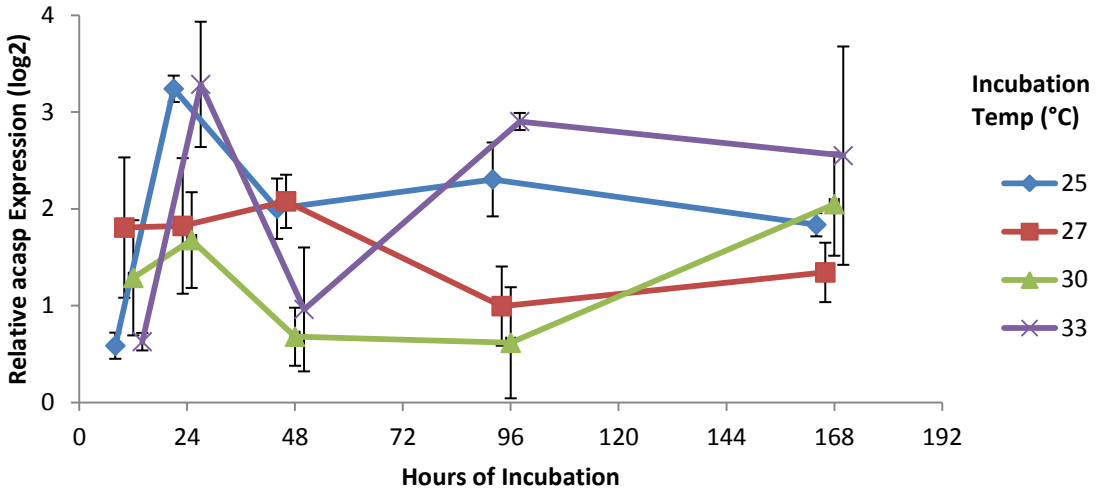

Figure 2. Expression of acasp in *Aiptasia* vs. time in control and elevated temperature incubation. Time points slightly offset for clarity. Values are means ± SD (n=3).

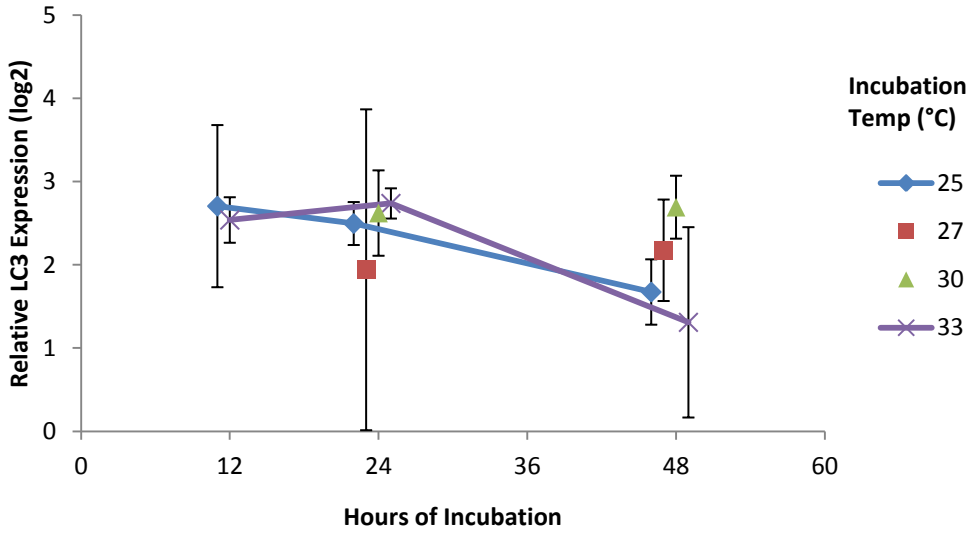

Figure 3. Expression of LC3 in *Aiptasia* vs. time in control and elevated temperature incubation. Time points slightly offset for clarity. There was a smaller data set for LC3 because some of the data were lost, including all time points past 48 hours. Values are means ± SD (n=3).

Imaging of autophagosome formation in mouse cells transfection with Human and *Aiptasia* LC3

To determine if the *Aiptasia* LC3 is similar in function to human LC3, both sequences were inserted into mouse cells using an EGFP vector to look for the formation of autophagosomes. The cells were imaged using confocal microscopy. Heterologous mouse cells containing empty EGFP vector had diffuse staining throughout the cytoplasm (Figure 4A). In contrast, heterologous mouse cells containing the human LC3 and *Aiptasia* LC3 sequence displayed punctate spots of bright fluorescence, suggesting the presence of autophagosomes (Figure 4B, 4C). This supports the hypothesis that *Aiptasia* LC3 functions in autophagy.

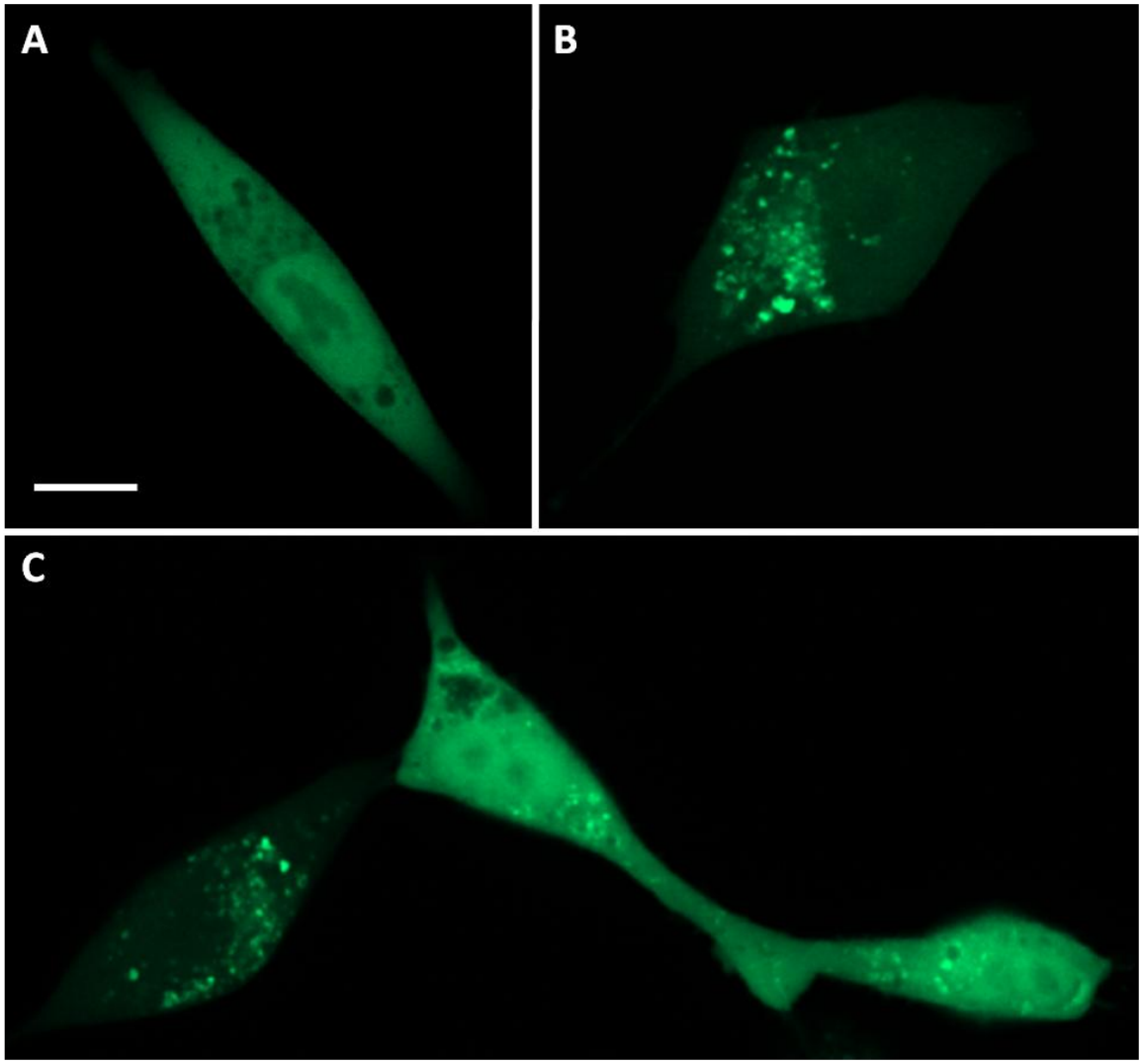

Figure 4. *Aiptasia* LC3 causes autophagosome formation in a heterologous mouse cell line. Confocal images of NIH3T3 cells transfected with (A) EGFP vector only (negative control), (B) human LC3 (positive control), (C) *Aiptasia* LC3. Autophagosomes are evident in human-LC3 and *Aiptasia*-LC3-transfected cells as bright punctate spots. Fluorescence excitation: 488/505-503nm. Scale bar = 10µm.

Discussion

Despite the significant effects of incubation time ($P = 3.65x10^{-7}$) and temperature ($P = 2.25x10^{-4}$) for the expression of acasp, there is no clear pattern to indicate whether expression of apoptosis genes was stimulated under increasing levels of thermal stress. These results could conflict with previous reports of increased apoptotic activity after elevated temperature incubation (Dunn et al. 2004, Dunn et al. 2007b). However, induction of apoptosis may occur post-translationally at the protein level. In mammalian systems, caspases are present as zymogens in the cell, awaiting activation (Kitazumi and Tsukahara 2011). Therefore in *Aiptasia*, acasp transcription may not be upregulated during induction of apoptosis in the host.

There was a downward trend in LC3 expression over time at 25 and 33°C, however neither change with incubation time ($P = 0.11$) nor temperature ($P = 0.60$) were significant. From evidence of increased bleaching in sea anemones treated with an autophagy inducer (Dunn et al. 2007a), a reasonable hypothesis would be that *Aiptasia* under thermal stress would upregulate LC3, suggesting activation of autophagic pathways. Since a downward trend was observed, this could indicate that autophagy is not the predominant mechanism for eliminating symbionts during a bleaching event. Dunn et al. (2007a) reported that when both apoptosis and autophagy were inhibited in *Aiptasia* under thermal stress, levels of bleaching were reduced; when either pathway was inhibited separately, there was no reduction in bleaching. This supports the possibility that autophagy could be a secondary cellular pathway to elicit a bleaching response. The results from this study are inconclusive in part because some of the data were lost and in

part because if low statistical power. A more complete analysis is needed to determine how thermal stress effects the expression of LC3.

Since data were not collected on the amount of algae that were expelled or retained in host tissues at each time point during incubations, the amount of bleaching that occurred was not quantified. However, the incubation temperatures are consistent with previous studies where significant bleaching of *Aiptasia* had occurred at 33°C after 24 hours of incubation (Dunn et al. 2004, Perez and Weis 2006)

Heterologous mouse cells containing the human and *Aiptasia* LC3 sequences exhibited punctate fluorescent spots, suggesting the presence of autophagosomes. Since LC3 is a common marker of autophagy, because of its localization to the autophagosome membrane, this suggests that *Aiptasia* LC3 functions in autophagy, thereby indicating the presence of autophagic machinery in the anemone. This evidence is supported by increase of autophagy with inducers (Dunn et al. 2007a), and the identification of LC3 homologues in other cnidarians, such as the anemone *Nematostella vectensis* (Sphilka et al. 2011) and visualized in *Hydra* (Chera et al. 2009). The untreated cells used in this study would not be expected to undergo autophagy. However, for imaging, the cells had to be transported between buildings. This transportation may have triggered a stress response and induced autophagy in the cells.

Further work is necessary to determine the links between apoptosis and autophagy, and how these cellular pathways play a role during bleaching. Research into apoptosis could focus on the post-translational regulation of acasp in *Aiptasia* to determine how apoptosis is regulated following thermal stress. Further examination of LC3 expression after thermal stress in *Aiptasia* will help determine possible roles

autophagy plays during bleaching. The interaction between apoptosis and autophagy is also unknown, so further descriptions of the cellular pathways leading to bleaching, along with molecules that bridge the two would be beneficial. Determining the cellular pathways that result in cnidarian bleaching is important for understanding bleaching events on coral reefs world-wide and whether and how corals will survive in a warming planet.

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